Electron Microscopy of Collagen*

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The principal previous electron microscopic observations on collagen have been made by Schmitt and his collaborators.[1-6] In their experiments collagen from several tissues and from vertebrate and invertebrate animals was used. They showed that collagen generally may be dispersed by teasing or by mild chemical treatment into transversely banded fibrils. The most probable spacing of the bands was 644 Ångström units (1 A = 10^{-8} cm.), identical with the X-ray diffraction periodicity discovered by Bear[7] in intact, naturally oriented, collagenous tissue. Imperfect periodicity in the micrographs was explained satisfactorily as the consequence of distortion during preparation of the specimen. Fibril width was random. Longitudinal cleavability of certain collagen fibers, notably those of rat tail tendon, was so pronounced that filaments were found approaching in width the resolution of even an electron microscope. The same collagen in weak acid, dispersed to the extent that it passed through a filter paper and contained no filaments discernible by any microscopic means, was reconstituted into the characteristic banded fibrils by raising the pH or ionic strength. Selective adsorption of heavy metal ions revealed at least five components in each repeating unit of the collagen fibrils. The fibrils were not noticeably affected by heating in water above the normal shrinkage temperature. No model has yet been proposed that explains the diffraction and electron microscopic results in terms of the polypeptide molecules of collagen.

In the present paper are reported electron microscopic observations on dispersed collagen obliquely coated with gold to reveal height and contour; on aqueous and nonaqueous dispersions of collagen; the fine structure of the bands in stained fibrils; and effects produced by heat, alkali, a neutral electrolytic swelling agent, and tannage on collagen fibrils. Most of the accompanying micrographs are reproduced at their original magnification, which was usually about 11,000 diameters. The scale drawn on the micrographs when not otherwise marked indicates a length of 1 micron (0.001 mm.). The collagen was obtained from beef hide and *ligamentum nuchae,* sharkskin, rat tail tendon, and surgical gut. Dispersion of the several tissues was effected in a Waring blender, and the collagen fibrils were isolated by repeated suspension and centrifuging.

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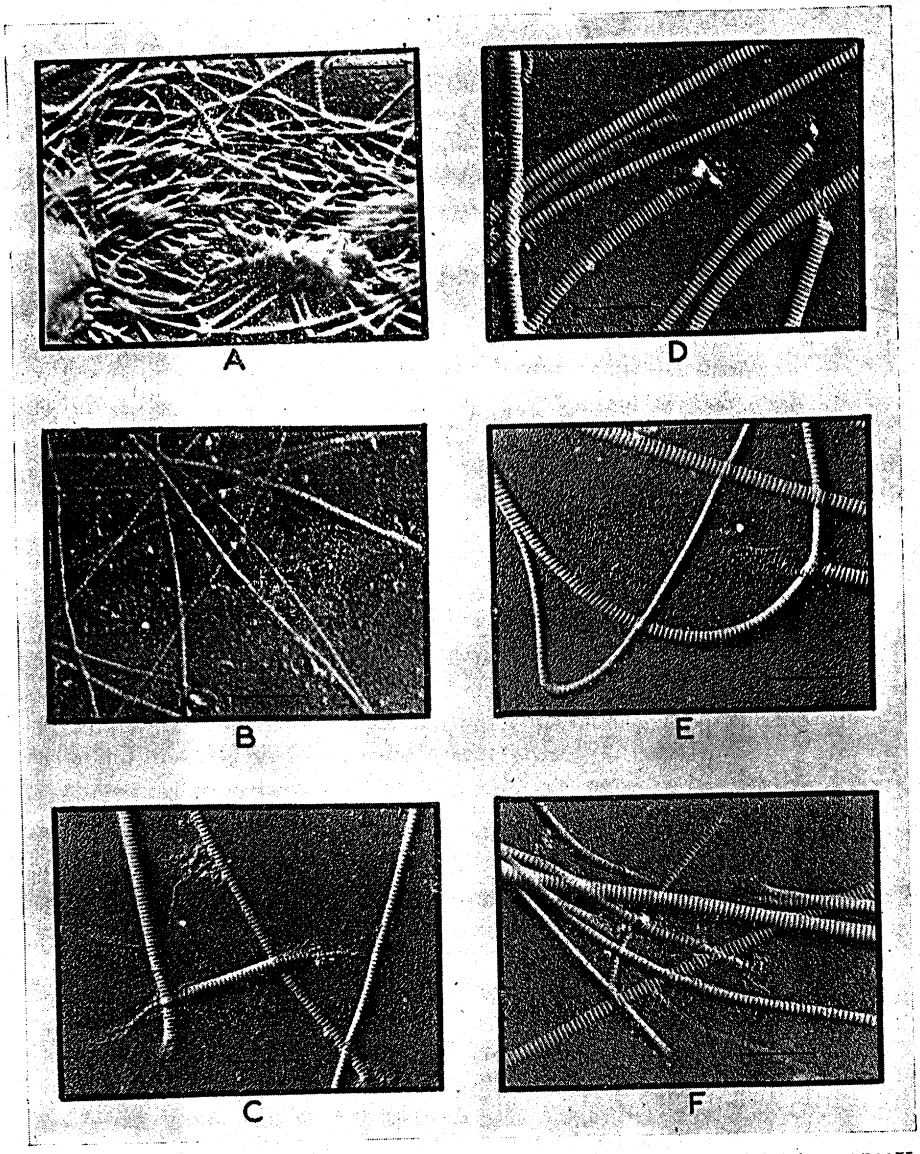

FIGURE I. Electron Micrographs of Gold-Shadowed Collagen. Magnification 11,300X. A and B, collagen from bovine *ligamentum nuchae;* C, D, E, and F, collagen from cowhide corium.

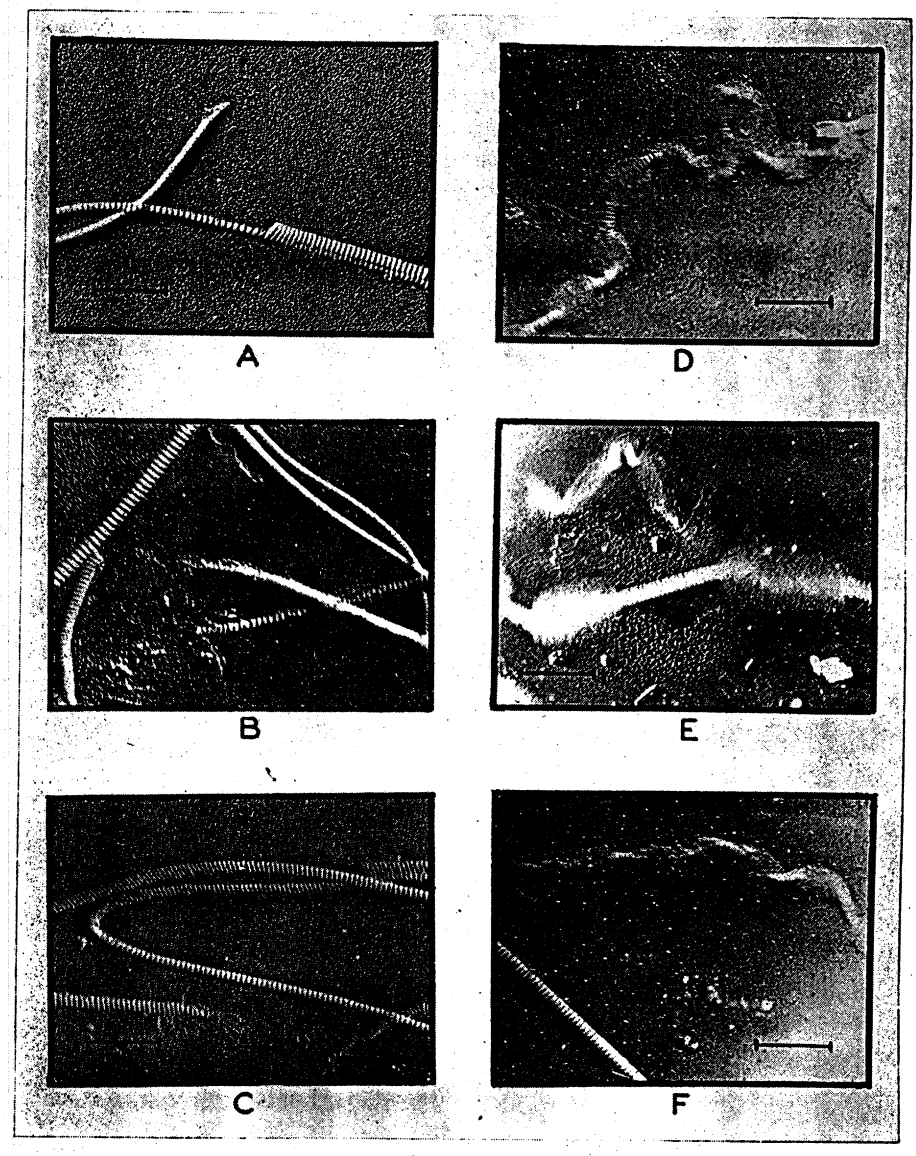

FIGURE II. Gold-Shadowed Fibrils of Cowhide Collagen. Magnification 11,300X. A, B, and C, native collagen; D, E, and F, collagen swelled in barium chloride solution before dispersion.

Gold-Shadowed Collagen Fibrils

All the micrographs in Figures I and II were obtained from gold-shadowed specimens. To prepare a specimen, an aqueous suspension was evaporated on a glass microscope slide, the slide transferred to a vacuum chamber and there coated with gold to a thickness of about 10 Å. The slide was dipped in collodion solution, and when dry the composite of collodion film, collagen and gold was floated off the slide on water and mounted conventionally for the microscope. The reproductions are negative prints of the original micrographs. Thus areas that are light indicate interception of an abnormal quantity of gold because of greater height than adjacent areas that lie in shadow. Background graininess is the result not of roughness of the glass slide surface but of residual gas in the chamber during evaporation and of aggregation of gold on exposure to the electron beam in the microscope.

Figures I A and B are micrographs of collagen from *ligamentum nuchae*. Figure I A shows a tangle of fibrils incompletely separated from the parent fiber bundle. Figure I B shows the fibrils at an early stage in the purification. Particulate debris in the background and on the fibrils obscures much of the detail, although rudimentary striations are apparent. Figures I C, D, E, and F, and Figures II A, B and C are micrographs of cowhide corium fibrils substantially free of noncollagenous substances.

From these micrographs it is apparent that the striations arise in a regular progression of raised and depressed elements along the fibrils. The striation period was determined from the length occupied by a group of at least ten striations. In Figure I D the period ranges between the limits 645 and 745 Å, with a pronounced maximum in the curve of frequency versus period at 650 to 675 Å. The period distribution from about 100 measurements on fibrils from the same brine-cured cowhide corium is shown in Figure III. The distribution is somewhat sharper than was measured by Schmitt, Hall and Jakus.[2] The position of the maximum, at about 660 Å, is in reasonable agreement with their value and that of Bear[7] from diffraction. It seems fair to conclude that disintegration in water in the blender is a properly mild procedure for separating the fibrils. The most prominent fibril in Figure II B is distinctive in having an almost uniform and abnormally high spacing, 810 Å.

The duplex nature of the ridges is clear at several points in Figures I D, E and F, and II A. In enlargements, fine structure in the hollows also may be seen occasionally, but the resolution of the gold-shadowed materials has not yet permitted a definite statement concerning the number and the position of these subsidiary bands. In many fibrils the angle of the striations with respect to the long axis departs so regularly and so much from 90° that a spiral construction is suggested. This possibility may be dismissed because the fibrils can easily subdivide longitudinally and because diffraction effects

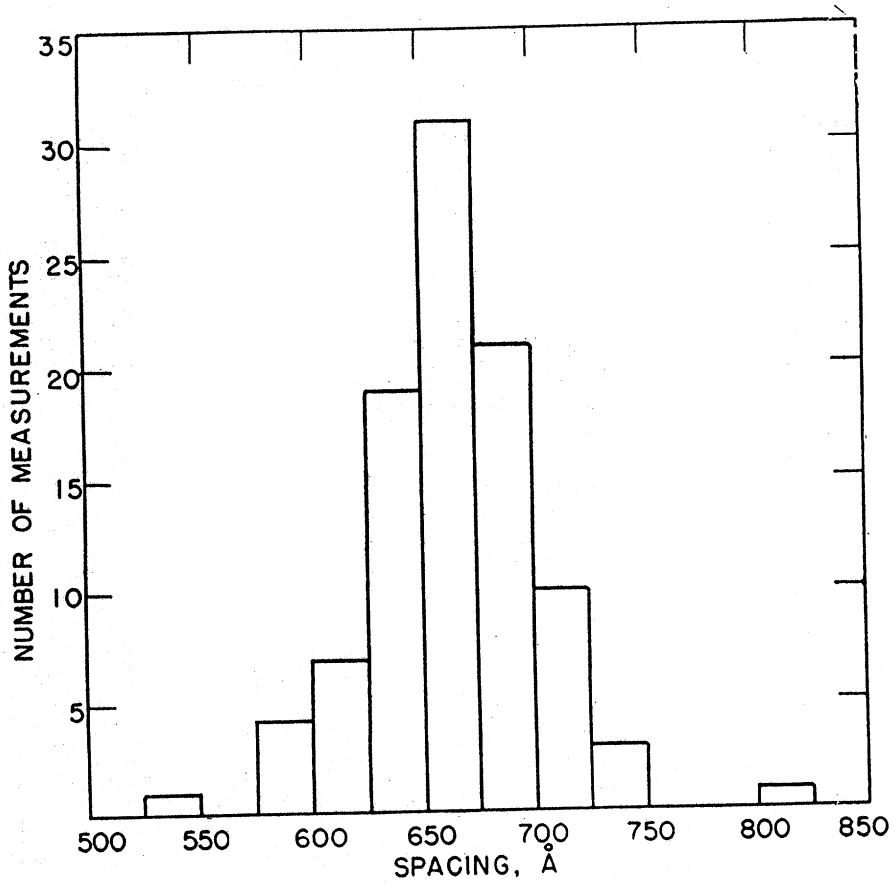

FIGURE III. Variation in the Spacing of the Fibril Transverse Striations of Cowhide Collagen Mechanically Dispersed in Water.

indicate that most of the diffracting material is arranged substantially parallel to the fiber axis. In Figure II A the widest fibril shows both the normal striations perpendicular to the axis and the less common tilted striations produced by relative displacement on opposite sides of the fibril. This figure illustrates also "in phase" and "out of phase" association of a pair of serrated fibrils. In phase association is shown well in Figure I E.

The extent to which cowhide fibrils fray under the dispersion conditions mentioned is typified in Figure I C. Separation of smaller threadlike units is shown, but to a much less degree than Schmitt has reported for rat tail tendon collagen.

From a shadow length it is possible to compute the height or thickness of the fibril casting the shadow, and then by comparison with the width to describe the form at least roughly as circular, oval, ribbonlike, and so forth

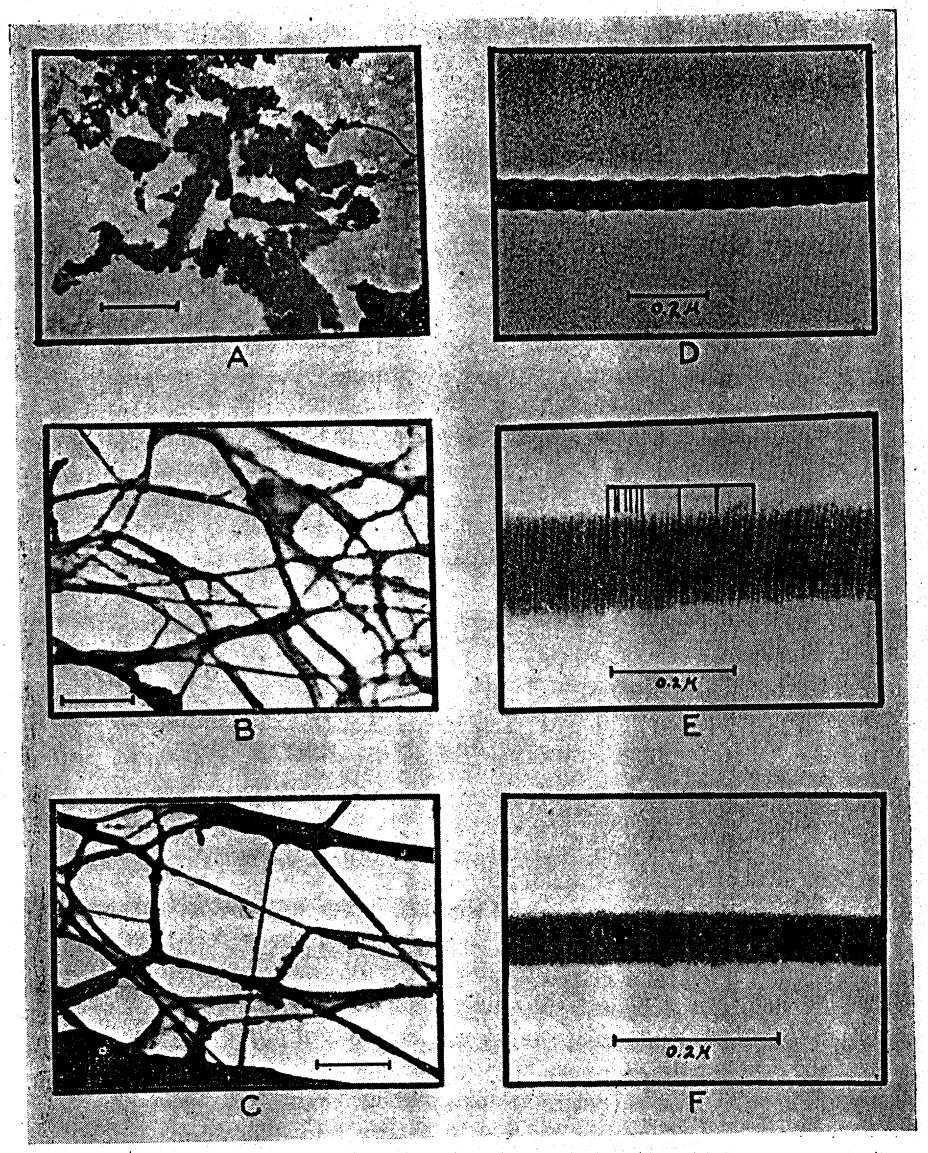

FIGURE IV. Electron Micrographs of Collagen. A, rat tail tendon dispersed in butanol, 11,300X; B and C, chrome tanned cowhide, 11,300X; D, formaldehyde tanned cowhide, stained with phosphotungstic acid, 61,000X; E, cowhide collagen stained with phosphotungstic acid, 88,000X; F, formaldehyde tanned cowhide stained with uranyl acetate, 124,500X.

For precise measurements of height, a long shadow is desirable, and th necessitates a small angle of the molecular beam of gold with respect to th specimen on its glass substrate in the evaporator. In our experiments th angle has been 10°, so the height of a specimen is tan 10° = 0.17 times th length of the shadow it casts. Proper measurement of the shadow can b made only for such fibrils or fibril elements as were perpendicular to th gold beam. Under these conditions shadows are cast on the background rath than on the fibrils, with the result that striations do not appear. No excep tionally good example is included in Figures I and II, which were select primarily to display the striations. The best example shown is in Figure I] The fibril in question is oval, the ratio of width to height being 3.5. Oth measurements, made on better defined shadows from 0.1 micron wide fibri of formaldehyde tanned cowhide, gave a width-to-height ratio of slightl less than 2. Portions of fibrils that are exceptionally flattened appear in Fig ures II C and I E and F.

Serrations in the shadows prove that the fibrils have a profile composed o an even succession of ridges and valleys. Again from measurements on th shadows the height of the ridges is computed to be roughly 15 per cent o the total fibril height. No good example of a serrated shadow appears the figures.

Fine Structure in the Transverse Bands

Subsidiary periodicity within the principal repeating unit is illustrat also in Figure IV E. This micrograph shows a pair of cowhide fibrils stain with 1 per cent phosphotungstic acid. There is no evidence that the sta appreciably affects the magnitude of the principal period. The large peri in the upper fibril is 635 Å, and the component bands occur at 0, 120, 19 300, 415, and 510 Å. The intervals are not equal, being 120, 75, 105, 115, 9 and 125 Å. Figure V B is a plot of the band positions. Both the intensity, indicated by the height, and the width are necessarily only roughly estimate Excluding the 120 Å band from consideration, an almost constant interv of 210 Å between alternate components is shown. The 120 Å and 195 Å ban are frequently not resolved, and appear as a single band with center ne 160 Å. The double ridges of the gold-shadowed fibrils are spaced as in Figu V A, and the relationship to the stained fibrils is evident on comparison wi V B. Data for Figure V A were obtained from an enlargement of Figure I] The periodicity is 660 Å, with minor intervals of 405 Å and 255 Å.

A specimen of formaldehyde tanned cowhide, dispersed in water a stained with 1 per cent uranyl acetate at pH 4.4, had five bands, at 0, 14 240, 345, and 475 Å within the major period, 585 Å (Figure V D). Aga excluding the second component, at 145 Å, an interval of about 230 Å is fou between alternate bands. On proportional expansion of the intervals of V to the extent that the total period is 635 Å, as for the nontanned collag

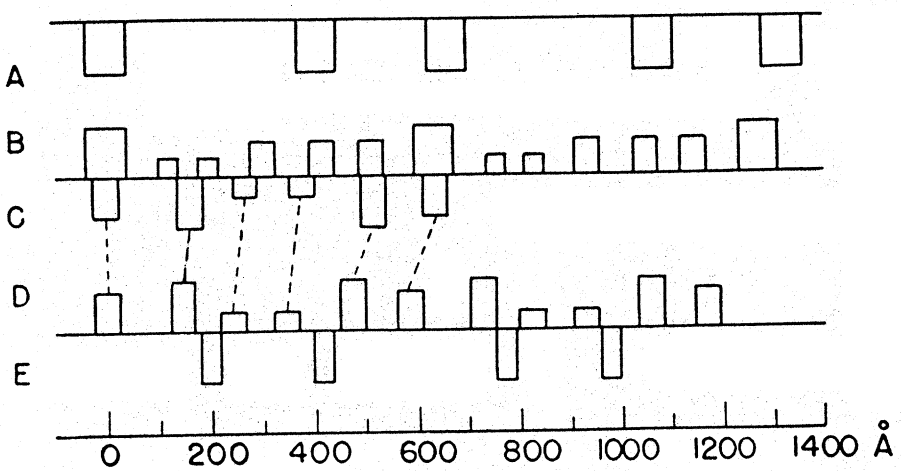

FIGURE V. Position of the Fibril Striations of Cowhide Collagen. A, principal ridges in gold-shadowed specimen; B, fibrils stained with phosphotungstic acid; C, formaldehyde tanned collagen stained with uranyl acetate. Measured spacings increased proportionally to make major period 635 Å as in B; D, formaldehyde tanned collagen stained with uranyl acetate; observed spacings; E, position of the light bands in same specimen as D.

of V B, the diagram V C is obtained. This indicates a slight relative displacement of two of the components (240 Å and 345 Å), as well as a considerable contraction of the structure as a whole. Distribution of uranyl acetate in the tanned collagen is such that the most prominent feature in the micrograph (Figure IV F) is the pairs of light lines, corresponding to areas containing little stain. The position of the white bands with respect to the less evident dark bands of higher scattering power is diagrammed in Figure V E.

Whether the increase in contrast brought about by staining indicates preferential combination with particular amino acid residues that are concentrated in the dark bands cannot be stated definitely at this time. This is not necessarily indicated, since micrographs of shadowed fibrils prove that the greatest thickness of scattering matter occurs in approximate coincidence with areas of greatest affinity for the stain. Uniform distribution along the polypeptide chain of chemical groups that react with the heavy-metal stain would then result in accentuation of light and dark bands simply because the bands vary in thickness. The configurations that the polypeptide assumes in forming the recurring thick and thin regions, and the explanation for the configurations in terms of the constituent amino acids, likewise are unknown. It is not necessary to stain or shadow the fibrils to demonstrate multiple structure within the bands; such structure has been observed several times in stretched unstained fibrils of collagen with ash content as low as 0.03 per cent.

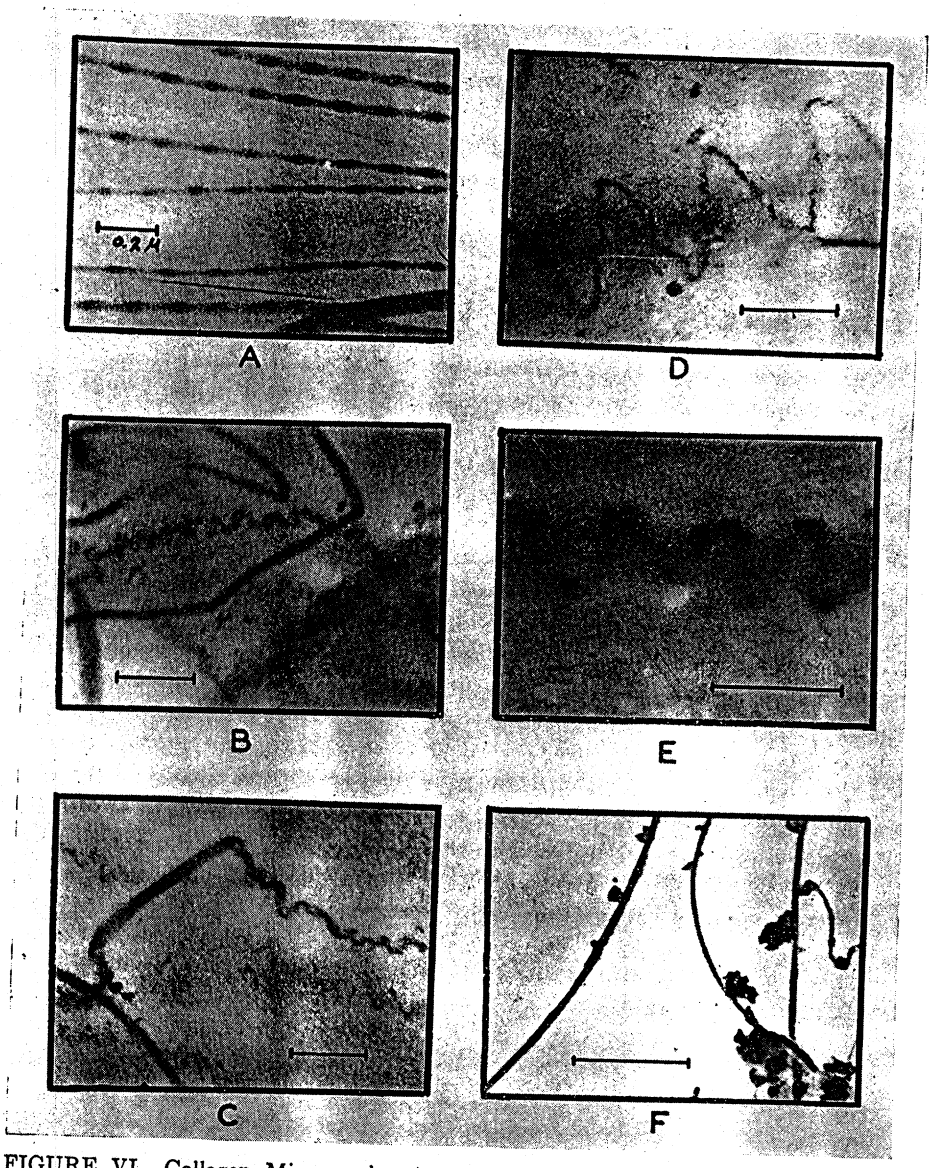

FIGURE VI. Collagen Micrographs. A, cowhide fibrils, showing supernormal band spacing, 44,000X; B and C, limed cowhide, 11,300X; D, sharkskin, shrinkage temperature, 40° C., dispersed in water, 14,400X; E, sharkskin, same preparation as D, 19,000X; F, vegetable tanned sharkskin, 17,200X.

It may be appropriate to remark in this place that microscopic demonstration of orderly arrangement in collagen fibrils at distances as little as 100 Å virtually precludes the existence in this material of regions that are "amorphous" and "crystalline" in the sense currently used in polymer physics. Rather, since demonstrable microscopic regularity extends down to distances about as small as the minimum size of an X-ray diffracting unit, it seems necessary to regard all the substance as crystalline and the orderliness of the spatial packing as everywhere less than perfect.

Effect of Swelling Agents on the Fibrils

The gelatinizing effect of a neutral electrolyte, barium chloride, is displayed in Figure II D, E and F. Cowhide corium cubes about 6 mm. on an edge were soaked for one week at room temperature in a saturated solution of barium chloride. The cubes were partially disintegrated in the blender and the collagen washed repeatedly by decantation to remove the electrolyte. Dispersion for the microscope was then effected by 10 minutes' treatment in the blender. The maximum temperature attained was little more than 30° C. Micrographs made from the dispersion demonstrate a remarkably localized attack on the fibrils by barium chloride. Enormous swelling has taken place, producing a material sufficiently fluid to spread into a film on drying. The film areas are so thin as to cast no measurable shadow, and contain no remnant of fibril organization. In the adjacent, striated fibril segments, the periodicity is not far from normal, with the largest number of measurements between 600 and 625 Å. The tendency of the swollen fibrils to loop (Figure II D, E) and twist (Figure II F) is characteristic.

Prolonged liming degraded cowhide fibrils as shown in Figure VI B and C. A flesh split of cowhide was kept for two months in saturated lime. It was then cut into small cubes, washed several times, partly disintegrated, washed several times more, dialyzed against distilled water, and finally dispersed in the blender. In the micrographs no detail is sharp. Where striations do appear, the spacing is normal. The most noteworthy feature is the dissolution of a fibril into an irregularly coiled major filament or a group of intertwined lesser filaments.

Fibrils of sharkskin collagen had an analogous appearance. The specimen was the dry-salted skin of a soup-fin shark stored for four years at room temperature. It showed no sign of deterioration or spoilage. The skin was softened in saturated brine, and the corium separated, washed and dispersed in distilled water. The fibrils were swollen, faint and indistinct, and commonly spiriform as in Figure VI D and E. The shrinkage temperature of the desalted sharkskin was 40° C., indicating much readier swelling by water than, for example cowhide, which has a shrinkage temperature of about 68° C., and which gives normal, striated fibrils when dispersed in water near room temperature. A commercial vegetable tanned sharkskin (shrinkage

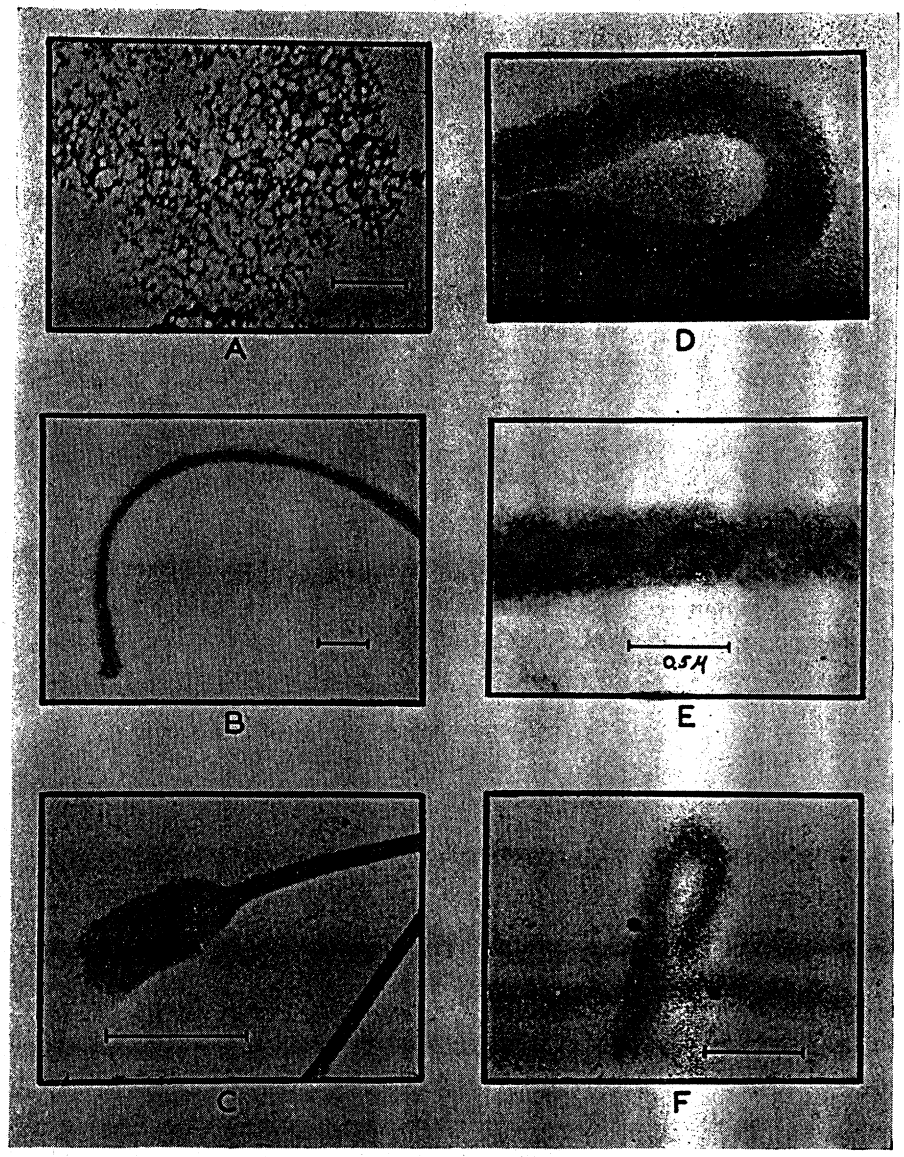

FIGURE VII. Electron Micrographs of Elastin and Collagen. A, elastin from *ligamentum nuchae,* 11,300X; B-F, cowhide collagen, water dispersion heated for five minutes; B, at 67° C., 7,300X; C, at 67° C., 21,000X; D, at 77° C., 21,000X; E, at 74° C., 31,000X; F, at 100° C., 15,200X.

temperature, 65° C.) fibrillated as in Figure VI F. The fibrils are not noticeably swollen or deformed, and striations are easily visible. The irregular dark material is common in the preparation, and is of undefined nature. It may be surmised to be a complex of tannin and collagen, so little swollen by water that longitudinal cleavage is not the usual course of subdivision. The necessity of a swelling (aqueous) medium for dispersing untanned collagen into fibrils is demonstrated by Figure IV A. This micrograph of rat tail tendon disintegrated in *t*-butanol gives little hint of the fibrous nature so readily manifested in water. The same absence of fibrillation was observed for shark-skin corium disintegrated in 90 per cent ethanol.

Hydrochloric or acetic acid, when added to an aqueous cowhide collagen dispersion until the acid concentration was about 0.05 *N*, swelled the fibrils considerably. The acid promoted general longitudinal subdivision, but even after a day's contact, striations were readily traceable across the groups of filaments that partially separated from the parent fibrils. The distribution in spacing was broadened and shifted toward subnormal values. Measurements to date have shown the most frequently occurring interval to be about 540 Å.

Figures VII B to F illustrate the effect on the fibrils of heating an aqueous collagen dispersion. Corium from cowhide of shrinkage temperature 68° C. was partly disintegrated in water, and the behavior of the fibers observed at 75 diameters on a heating stage. Sudden shrinkage of the smallest fibers was noted at 63° C. As the temperature was raised, the larger fibers shrunk progressively. At 67 to 68° C., all had shrunk, and the dispersion was considerably clarified by agglomeration of the particles. The unheated collagen suspension was dispersed further for the electron microscope. Portions of this very dilute dispersion were heated for five minutes at 63, 67, 74, 77, and 100° C., then cooled to room temperature, and portions evaporated on a collodion film as usual. The 63° C. specimen was strongly reminiscent of barium chloride-treated collagen. For the most part, the fibrils were striated, but at irregular intervals along the fibrils, particularly at fibril ends, enormous swelling and flattening occurred in the dried specimen. The distribution in periodicity was somewhat broadened, and shifted to smaller values. The lowest value measured was 450 Å. At 67° C., the usual fibril appearance was that shown in Figure VII B, although some (Figure VII C) more nearly resembled the average fibril heated to 63° C. Above about 75° C. (Figures VII D, E, F), all fibrils were swollen completely and were distinguished by their relatively short length as compared with unheated fibrils. It has not been determined whether this indicates actual breakup into short segments or association of the longer particles into bodies so large that they segregated and did not comprise any part of the sample examined. The appearance of a swollen fibril is that of a thread in the form of a loose, flattened spiral, embedded in a thin, transparent film much wider

than the diameter of the spiral. As the temperature is raised, the convoluted core becomes less prominent, and the film becomes thicker and more opaque. The reaction appears to be one of progressive, irreversible gelatinization of the collagen.

From present evidence, the shrinkage of collagen may be described as follows: Initially the units are orderly arrays of linearly extended collagen molecules, the striated fibrils. When plasticized by heat and water or other swelling agent, the interactions maintaining the orderly structure are weakened, and the natural contractile tendency (due to entropy) of long, flexible molecules becomes predominant, shortening the fibril as a whole. Close analogies exist in the shrinkage of silk by acid and the shrinkage of crystallized rubber by heating. The film, one supposes, is the residue of collagen molecules that were able to separate most easily from the parent fibril because of proximity to its surface. Neither in the film nor in the core is there any trace of the striations.

Tannage

Like the vegetable tanned sharkskin mentioned above, chrome tanned cowhide fibrillated imperfectly in the blender (Figures IV B, C). The presence of chromium was easily detected because of its high scattering power. Regular striations are not plainly visible in the micrographs reproduced because of nonuniform deposition of the bulk of the chromium. Although the chrome liquor does stain the collagen analogously to phosphotungstic acid or uranyl acetate, the predominant amount of chromium absorbed appears as small lumps or nodules within fibril bundles.

Cowhide, formaldehyde tanned at pH 7 to 8, exhibited fibrillation as perfect as that of untanned cowhide. Formaldehyde tanned fibrils had the usual striated aspect, but the periodicity was generally low. The spacing in Figure IV D is 570 Å; in IV F it is 585 Å. On tanning, the regular collagen structure appeared to contract several per cent without noticeable distortion. The distribution curve of spacing suggests by its form that all the fibrils were not uniformly tanned. The extent to which the periodicity varies with the formaldehyde content of the leather has not been measured. A limited number of measurements on canaigre tanned cowhide gave a most probable spacing of 575 to 600 Å, indicating that this tannage, too, appreciably shrinks the fibrils.

As expected, a general result of tanning is to raise the temperature at which thermal deterioration of the organized collagen fibrils sets in.

Experiments with Stretched Tissue; Elastin

Experiments thus far have not revealed a method by which the periodicity of the collagen fibril may be increased controllably. Occasionally large spacings, such as in Figure VI A, are observed, but only when breakage of the

collodion film places unusual stress on the fibrils. Stretching collagenous materials orients the fibers in the direction of pull, as is evidenced by the development of an X-ray fiber pattern. It apparently does not, however, stretch the fibrils, for the striations in dispersed plain surgical gut appear within the normal limits of spacing. Fine structure in the bands of this material has also been observed. These results are in harmony with the diffraction effects reported by Bear.[7]

Preparations of *ligamentum nuchae* were made to discover whether elastin, the protein of yellow connective tissue, is structurally similar to collagen. *Ligamentum nuchae* is composed of roughly two-thirds elastin and one-third collagen. The elastin occurs as fibers approximately 5 microns wide and these are readily distinguished from the collagen fibers by staining with orcein. To prepare material for the electron microscope, a portion of a ligament was macerated in water in the blender, and the suspension passing a 325-mesh screen was made tenth normal in sodium hydroxide and boiled for ten minutes to destroy the collagen. The pH was reduced to 6, and the suspension dialyzed, then frozen to coagulate the elastin. The elastin was centrifuged, washed to separate gelatin, and finally dispersed in water. The separated elastin stained red with orcein and showed no trace of collagen with an aniline blue-phosphotungstic acid stain. A typical electron micrograph of the elastin is reproduced as Figure VII A. Only a reticular structure, devoid of the fibrillation characteristic of collagen, is shown. As alternative procedures for removing collagen from the *ligamentum nuchae* dispersion, extraction with 40 per cent urea and digestion with pepsin were tried. Neither procedure was completely successful. Collagen fibrils appeared in the micrographs, somewhat obscuring the elastin. The elastin, however, had much the same netted aspect, indicating that the original sodium hydroxide treatment had not harmed the elastin. Our finding that elastin, though present as fibers in the ligament, is reticular on smaller subdivision makes more readily understandable Astbury's observation[8] that stretched elastin gives a diffraction pattern characteristic of an unoriented, structureless material.

Summary

Collagen from beef hide and *ligamentum nuchae*, sharkskin, rat tail tendon and surgical gut was mechanically dispersed in water. The fibrils produced had transverse striations arising in a regular progression of raised and depressed elements along the fibrils. The normal striation period of approximately 660 Å was considerably reduced by tannage and by dilute acid; it has not been controllably increased. A maximum of six components, not equally spaced, were distinguished in the principal period. Dispersions of collagen with low shrinkage temperature (a sharkskin corium, cowhide soaked in saturated barium chloride solution or limed for a long period) showed marked, irreversible deterioration of the regular fibril structure.

Fibrils of normal cowhide collagen heated above the shrinkage temperature swelled greatly, shortened, and lost their striations. The shrunken fibril appeared as a convoluted core embedded in a transparent film. Fibrillation of a chrome tanned cowhide was imperfect. The chromium was nonuniformly distributed in the fibrils. In a nonswelling medium (butanol), collagen did not fibrillate but disintegrated into irregular fragments. Elastin was separated from *ligamentum nuchae* only in reticular form.

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